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Puumala Virus and Two Genetic Variants of Tula Virus Are Present in Austrian Rodents

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Puumala and Tula viruses are hantaviruses found in Europe and are associated with the rodents *Clethrionomys glareolus* and *Microtus arvalis,* respectively. Puumala virus is associated with the human disease nephropathia epidemica. In Austria, ten clinically diagnosed cases of nephropathia epidemica, presumably caused by Puumala virus infection, have been reported but not virologically confirmed [Leschinskaya et al., 1991; Aberle et al., 1996]. To identify the hantaviruses that are present in Austria, five species of rodents were trapped and screened for virus antibodies, antigen, and RNA. Hantaviruses were detected in two species, *Cl. glareolus* and *M. arvalis,* by reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR products from *Cl. glareolus* tissues yielded a unique Puumala virus sequence distinct from Puumala virus sequences reported from other parts of Europe. RT-PCR products from *M. arvalis* tissues yielded two genetically distinct Tula virus sequences, one similar to sequences reported from Slovakia and the Czech Republic and another that appears to be a novel genetic variant of Tula virus. This is the first confirmed report of hantaviruses in Austria. ***J. Med. Virol. 53:174–181, 1997.*** © 1997 Wiley-Liss, Inc.†

KEY WORDS: Bunyaviridae; Hantavirus; Microtus arvalis; Clethrionomys glareolus; PCR

INTRODUCTION

In Europe, hantaviruses are known to cause hemorrhagic fever with renal syndrome (HFRS). This syndrome is characterized by high fever, headache, backache, abdominal pain, nausea, vomiting, renal insufficiency, and hemorrhagic manifestations; the spectrum of clinical severity can range from mild disease to death [Lee, 1989; Lähdevirta, 1989]. Clinically severe cases of HFRS have been reported in Albania, Bulgaria, Greece, Romania, and the former Yugoslavia with mortality rates of 5% to 15% [Manasia et al., 1977; Eltari et al., 1987; Gligic et al., 1989; Vasilenko et al., 1990; Gligic et al., 1992; Avsic-Zupanc et al., 1994; Antoniadis et al., 1996]. This form of HFRS is caused by Dobrava/Belgrade (DOB) virus and possibly Hantaan (HTN) virus [Gligic et al., 1992; Avsic-Zupanc et al., 1994; Antoniadis et al., 1996]. Another form of HFRS, known as nephropatnia epidemica (NE), is endemic in Scandinavia, European Russia, and Western Europe and caused by Puumala (PUU) virus [Brummer-Korvenkontio et al., 1982; Tkachenko et al., 1982; Lähdevirta, 1989; Pilaski et al., 1991; Clement et al., 1994; Le Guenno et al., 1994; Rollin et al., 1994; Groen et al., 1995]. Though severe cases and deaths have been reported [Pilaski et al., 1994; Valtonen et al., 1995], NE is generally associated with milder hemorrhagic disease and a lower case-fatality rate (<1%), compared with HFRS resulting from DOB or HTN virus infection [Lähdevirta, 1989].

Hantaviruses (*Bunyaviridae*, genus *Hantavirus*) are enveloped viruses with a genome that consists of three single-stranded RNA segments designated small (S), medium (M), and large (L) [Elliott et al., 1991]. The virus genes are encoded in the negative (genome-complementary) sense. The nucleocapsid protein, envelope glycoproteins (G1 and G2), and RNA polymerase are encoded on the S, M, and L RNAs, respectively. Currently, 16 distinct hantaviruses are recognized which represent distinct serotypes or genotypes [Plyusnin et al., 1996].

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Rodents are the natural hosts of most hantaviruses. European rodent species that are known hantavirus reservoirs include *Apodemus agrarius* and *A. flavicollis,* the hosts of HTN and DOB viruses, respectively; *Clethrionomys glareolus,* the host of PUU virus; and *Microtus arvalis* and *M. rossiaemeridionalis,* the hosts of Tula (TUL) virus [Plyusnin et al., 1994]. TUL virus has been detected in *Microtus* spp. trapped in Russia, the Czech Republic, and Slovakia [Plyusnin et al., 1994; Plyusnin et al., 1995; Sibold et al., 1995]. A single human infection with TUL virus has been described [Vapalahti et al., 1996], but the virus is not known to cause human disease.

In Austria, ten clinically diagnosed cases of NE, presumably caused by PUU virus infection, have been reported [Leschinskaya et al., 1991; Aberle et al., 1996]. Antibodies to PUU virus have been detected in human populations by serosurveys in Carinthia and Lower Austria [Aberle et al., 1996]. In addition, a 5% prevalence of antibodies of PUU viruses has been found in Austrian cats [Nowotny, 1994], and hantavirus antigen has been detected in tissue samples from rodents and cats [Nowotny et al., 1994; Aberle et al., 1996]. While clinical and serologic data strongly suggest that PUU virus is endemic in Austria, the virus(es) causing HFRS have never been typed. The purpose of this study was to detect hantavirus infection in Austrian rodent species and to characterize enzootic hantaviruses. This report presents the first genetic evidence that PUU and TUL viruses are present in Austrian rodents.

MATERIALS AND METHODS

Rodent Trapping

Rodents were trapped in eight areas as described by Gelbmann [1996, Fig. 1]: (1) Korneuburg, federal state of Lower Austria; (2) Schönbrunn and (3) Veterinary University, federal state and city of Vienna; 4) Wels, federal state of Upper Austria; (5) Forst, (6) Preitenegg, and (7) Klippitztörl, Lavanttal Valley, federal state of Carinthia; and (8) Rankweil, federal state of Vorarlberg. Rodents were live-trapped in fall 1995, February 1996 (Schönbrunn site), and May 1996 (Preitenegg and Klippitztörl sites). The traps were placed in fields, forests, and at the edges of forests. *Mus musculus* were trapped inside buildings. At the Forst, Preitenegg, and Klippitztörl sites, traps were placed in forests near the residences or workplaces of clinically diagnosed NE cases [Aberle et al., 1996]. Captured rodents were euthanized by using carbon dioxide, weighed, measured, sexed, identified to species, and then necropsied. Blood samples were obtained by cardiac puncture. Lung, liver, spleen, and kidney samples were collected and stored at −70°C until processed for antigen or RNA. The identities of rodents later found to be positive for hantavirus antibody were confirmed at the Natural History Museum of Vienna.

Detection of Hantavirus Antibodies in Rodent Sera

To detect immunoglobulin G (IgG) antibodies to hantaviruses, rodent sera were screened by using a PUU virus IgG enzyme-linked immunosorbent assay (ELISA) kit (Progen Biotechnik GMBH, Heidelberg, Germany). Assays were performed as described in the kit protocol except that the positive control consisted of either a mixture of anti-PUU monoclonal antibodies or serum from an antibody-positive, antigen-positive *Cl. glareolus*. Both reagents were obtained from Dr. Stephan Aberle, Institute of Virology, University of Vienna. Serum from an uninfected laboratory mouse was used as a negative control. In addition, anti-mouse IgG peroxidase conjugate (Biorad Laboratories, Richmond, CA) was used in place of the anti-human IgG peroxidase conjugate provided in the kit. Anti-mouse IgG peroxidase conjugate has been used previously in an ELISA assay to detect hantavirus antibodies in *Clethrionomys, Microtus,* and *Myopus* spp. rodents [Niklasson et al., 1995] Antibody-positive animals were defined as those exhibiting fivefold or greater optical density values compared to the negative control.

Detection of Hantavirus Antigen in Rodent Tissues

To detect the presence of hantaviruses in rodents with hantavirus antibody, rodents that were positive by IgG ELISA were tested for hantavirus antigen in their lungs by indirect immunofluorescence assay. Five-micrometer cryocut sections of lung tissue were mounted on glass microscope slides, fixed in chilled acetone for 10 min, dried, and then incubated in convalescent serum from an Austrian NE patient [diluted 1:10 in phosphate-buffered saline (PBS), pH 7.4] for 45 min at 37°C. After three 5-minute washes in PBS, the sections were incubated with an anti-human-IgG-fluorescein isothiocyanate conjugate (Jackson Immuno Research, West Grove, PA, diluted 1:20 in PBS) for 45 min at 37°C. After three 5-minute washes in PBS, coverslips were mounted on the slides with glycerin:PBS (9:1) and the sections were examined with a fluorescence microscope.

RNA Extraction

To obtain RNA templates for reverse transcription-polymerase chain reaction (RT-PCR) amplification, RNA was extracted from lung tissue of antigen-positive rodents. A 50–100 mg piece of lung tissue from each rodent was ground in 1 ml of TriPure reagent (Boehringer Mannheim Corp., Indianapolis, IN) and then mixed with 200 μl of chloroform:isoamyl alcohol (24:1). After incubation for 15 min on ice, the mixture was centrifuged at 14,000 rpm, 4°C for 15 min. RNA was isolated from the aqueous layer by using an RNaid kit (Bio 101, Inc., La Jolla, CA) and eluted in 50 μl of nuclease-free water.

RT-PCR

To perform RT-PCR, oligonucleotide primers were designed that targeted conserved regions of the S RNA of PUU, TUL, and Prospect Hill hantaviruses. RT-PCR was carried out using an Access RT-PCR kit (Promega Corp., Madison, WI) and a Perkin-Elmer 9600 thermocycler (Perkin-Elmer Corp., Norwalk, CT). Each RT-PCR reaction contained 1X AMV/*Tfl* reaction buffer, 1 μM primer PPT334C (5′-TATGGIAATGTCCTTGATGT-3′; I = inosine), 1 μM primer PPT986R (5′-GCACAIGCAAAIACCCA-3′), 500 μM $MgSO_4$, 200 μM dNTPs, 5 U avian myeloblastosis virus reverse transcriptase, 5 U *Tfl* DNA polymerase, and 10% of the RNA from a single RNA extraction. Reverse transcription was carried out for 1 hr at 42°C, and the reactions were heated for 2 min at 94°C. The reactions were then subjected to 40 cycles of a temperature profile, consisting of 94°C for 30 sec, 45°C for 1 min, and 68°C for 2 min, followed by a final extension of 68°C for 7 min. Ten percent (5 μl) of each reaction was then electrophoresed on a 1% agarose gel in Tris-borate-EDTA buffer. After electrophoresis, the gel was stained with ethidium bromide, and PCR products were visualized by UV translumination to assess product yield. If no visible products were obtained in the RT-PCR reaction, second-round PCR was carried out using primers PPT376C (5′-CCIAGTGGICAIACAGC-3′) and PPT716R (5′-AAICCIATIACICCCAT-3′). The target regions for these primers were conserved regions of the S RNA of PUU, TUL, and Prospect Hill hantaviruses that resided totally within the amplified region of the RT-PCR primers in a "nested" fashion [Nichol et al., 1993]. Each second-round reaction contained 1X PCR buffer with 150 μM $MgCl_2$ (Boehringer Mannheim Corp.), 1 μM primer PPT376C, 1 μM primer PPT716R, 200 μM dNTPs, 5 U Taq polymerase (Boehringer Mannheim Corp.), and 1 μl of the first round reaction. The reactions were then subjected to 36 cycles of a temperature profile consisting of 95°C for 30 sec, 45°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 10 min. Product yield was assessed by agarose gel electrophoresis, as described previously.

Sequence Determination and Analysis

To obtain sequence data for each virus, the remaining RT-PCR or PCR reaction was electrophoresed on a 1% agarose gel in Tris-acetate-EDTA buffer. After being stained with ethidium bromide, specific PCR products were located by UV translumination, sliced from the gel, and purified from gel slices by using a Sephaglas Bandprep kit (Pharmacia Biotech, Inc., Piscataway, NJ). Dye terminator cycle sequencing reactions were carried out by using 5%–50% of the gel-purified product (depending on product yield), ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase FS (Applied Biosystems Inc., Foster City, CA), and 3.3 pM of primer PPT334C, PPT376C, PPT986R, or PPT716R. Extension products were purified by using Centri-sep spin columns (Princeton Separations, Inc., Adelphia, NJ) and sequenced on an ABI 373A automated DNA sequencer (Applied Biosystems Inc.). Chromatograms were analyzed by using Sequencher 3.0 software (Gene Codes Corp., Ann Arbor, MI) run on a Macintosh Quadra 800 (Apple Computer Corp., Cupertino, CA). Sequences were obtained from both strands of each RT-PCR product for verification.

Hantavirus sequences reported in this paper were deposited in the GenBank sequence database under the following accession numbers: PUU virus strains Kl1 (U95308), Kl6 (U95306), and P1 (U95307); and TUL virus strains K11 (U95305), K26 (U95310), K58 (U95311), K64 (U95312), O8 (U95313), O20 (U95304), O64 (U95312), O24 (U95302), and O52 (U95303).

To estimate phylogenetic relationship of Austrian hantaviruses to other known hantaviruses, the Austrian sequences were aligned with the corresponding nucleocapsid gene sequences of hantaviruses retrieved from the GenBank sequence data base (Fig. 2). Alignments were performed by using the PileUp program of the Wisconsin Package Version 8.1 UNIX (Genetics Computer Group, Inc., Madison, WI) run on a DEC 3000-500X AXP workstation (Digital Equipment Corp., Maynard, MA) with a gap creation penalty of 5.0 and a gap extension penalty of 0.3, followed by manual adjustment. Maximum parsimony analyses were performed using PAUP 3.1.1 [Swofford, 1993] run on a PowerPC 9500/132 (Apple Computer Corp). Bootstrap analyses [Felsenstein, 1985] were carried out using PAUP.

RESULTS

A total of 344 rodents were taken alive by trapping. Five species were represented: *Cl. glareolus*, *Microtus arvalis*, *A. flavicollis*, *A. sylvaticus*, and *Mus musculus*. *A. flavicollis* was the most frequently trapped rodent (105 specimens), followed by *Microtus arvalis* (78), *Cl. glareolus* and *A. sylvaticus* (56 each), and *Mus musculus* (49). Hantavirus antibodies were detected in 24 rodents; 11 others had borderline ELISA results (greater than threefold but less than fivefold optical density values compared to the negative control; Table I). All 24 rodents that showed hantavirus antibodies by ELISA and 11 with borderline ELISA results were tested for hantavirus antigen. Ten seropositive rodents were found to have hantavirus antigen in their lung tissues and 4 were questionable for antigen (Table I). RNA was extracted from the lung tissue of these 14 rodents and of two antibody-positive, antigen-negative rodents (one *A. flavicollis* and one *Mus musculus*) and tested by RT-PCR. RT-PCR products were obtained from two *Cl. glareolus* from Klippitztörl and one from Preitenegg (Table I). When the amplified products were sequenced, they each yielded a 611 nucleotide (nt) sequence that shared over 99% nt and amino acid sequence identity with each other and displayed a minimum of 14.9% nt and 3.5% amino acid sequence dissimilarity with other PUU viruses (Table II).

Four *M. arvalis* from Korneuburg and four *M. arvalis*

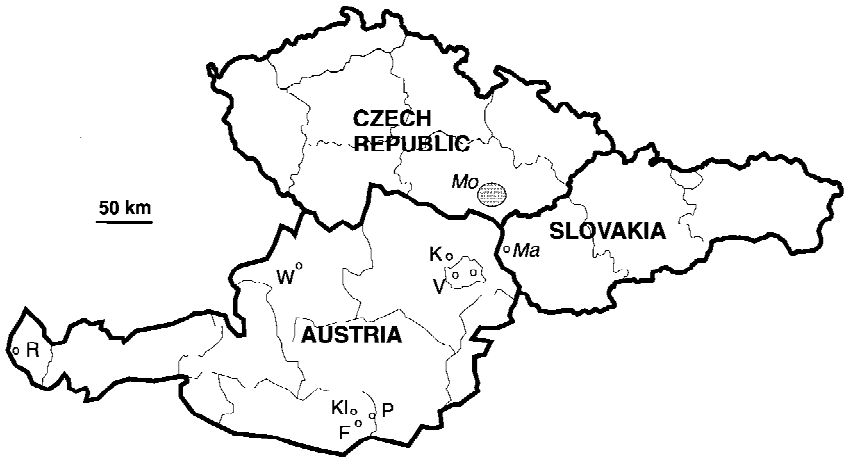

Fig. 1. Map of Austria showing rodent trapping sites and areas of Slovakia and the Czech Republic where TUL viruses have been detected. Trap site abbreviations are R (Rankweil), W (Wels), K (Korneuburg), V (Vienna, 2 sites), F (Forst), P (Preitenegg), and Kl (Klippitztörl). Areas where TUL virus strains have been detected previously are Ma (Malacky, Slovakia), and Mo (Moravia, Czech Republic, approximate area indicated by shaded circle). Distances are indicated by the scale bar.

TABLE I. Results of Antibody Detection, Antigen Detection, and RT-PCR

Site	Species	Antibody positive[a]	Antigen positive[a]	RT-PCR positive[a]
Korneuburg	*A. sylvaticus*	2[b]/42	1[c]/1	0/1
(Lower Austria)	*Cl. glareolus*	0/3	N.D.[d]	N.D.
	M. arvalis	4[c]/53	4[c]/4	4/4
Schönbrunn +	*A. flavicollis*	3[b]/34	0/1	N.D.
Veterinary	*A. sylvaticus*	0/1	N.D.	N.D.
University	*M. arvalis*	1/1	0/1	N.D.
(Vienna)	*Mus musculus*	6[c]/26	0/5	N.D.
Wels	*A. flavicollis*	1/32	0/1	0/1
(Upper Austria)	*A. sylvaticus*	0/9	N.D.	N.D.
	Cl. glareolus	0/9	N.D.	N.D.
	M. arvalis	6[b]/22	5[c]/5	5[e]/5
Forst +	*A. flavicollis*	1[c]/12	N.D.	N.D.
Preitenegg +	*A. sylvaticus*	0/4	N.D.	N.D.
Klippitztörl	*Cl. glareolus*	5/22	4[c]/5	3/4
(Carinthia)	*M. arvalis*	0/2	N.D.	N.D.
	Mus musculus	0/2	N.D.	N.D.
Rankweil	*A. flavicollis*	2[c]/27	0/1	N.D.
(Vorarlberg)	*Cl. glareolus*	0/22	N.D.	N.D.
	Mus musculus	4[c]/21	0/3	0/1

[a]No. positive or borderline/no. tested.
[b]Includes 2 borderline or questionable samples.
[c]Includes 1 borderline or questionable sample.
[d]Not determined.
[e]Includes one sample found to be positive by nested PCR only.

from Wels were positive by non-nested RT-PCR (Table I). In addition, a fifth *M. arvalis* from Wels was negative by RT-PCR, but positive when tested by nested PCR (Table I). When sequenced, each of the four RT-PCR products from Korneuburg yielded a 600 nt sequence. The four sequences were 100% identical to one another and displayed approximately 98% nt and 100% amino acid identity with TUL virus sequences from western Slovakia [Sibold et al., 1995; Table III). The RT-PCR products from the four *M. arvalis* from Wels also yielded 600 nt sequences, but these sequences, which were 98.8% to 100% identical to one another (Table III), represented a distinct variant of TUL virus. The Wels sequences displayed less than 93% nt identity with the sequences from Korneuburg and less than 94% identity with TUL virus sequences from Slovakia and the Czech Republic [Plyusnin et al., 1995; Sibold et al., 1995; Table III]. The nested PCR product (307 bp) obtained from an *M. arvalis* from Wels (O8) was 100% identical to 2 of the RT-PCR products from Wels. In the region of the TUL S RNA examined in this study, all Austrian, Slovakian, and Czech TUL virus sequences displayed a minimum of 18.2% nt and 4% amino acid sequence divergence when compared to TUL virus sequences from Russia (Table III and data not shown). Like the previously reported Slovakian and Czech TUL

TABLE II. Sequence Identity Among Austrian PUU Virus Strains and Between Austrian Strains and Strains From Other Countries

	Percent nucleotide (nt) and amino acid (aa) sequence identity													
	Austria[a]		Finland[b]		France[c]		Germany[d]		Russia[e]		Sweden[f]		Vranica[g]	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Austria	99.2–99.5	99.5–100	82.3–83.3	94.6–95.6	79.1–79.7	92.6–93.1	84.6–85.1	96.1–96.5	79.0–80.5	93.1–95.1	82.6–83.3	94.1–94.6	81.7–82.4	93.6–94.6

[a]Includes strains Kl1, Kl6, and P1.
[b]Includes strains Evo/12cg/93 (GenBank accession no. Z30702), Sotkamo (X61035), and PUU/Virrat/25cg/95 (Z69985).
[c]Includes strain PUU90-13 (U22423).
[d]Includes strain Berkel (L36943).
[e]Includes strains Udmurtia/894Cg/91 (Z21497), K27 (L08804), P360 (L11347), and CG1820 (M32750).
[f]Includes strain PUU/Virrat/25cg/95 (Z69985).
[g]Includes strain Vranica (U14137). This strain is reported to have originated in the former Yugoslavia but sequence analyses have suggested that it is of Swedish origin [Hörling et al., 1995; Reip et al., 1995].

TABLE III. Sequence Identity Among Austrian TUL Virus Strains and Between Austrian Strains and Strains From Other Countries

	Percent nucleotide (nt) and amino acid (aa) sequence identity									
	Austria[a] (Korneuburg)		Austria (Wels)[b]		Slovakia[c]		Czech Republic[d]		Russia[e]	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Korneuburg	100	100	92.3–92.7	100	97.7–97.8	100	96.3–96.8	99.5–100	80.7–81.7	95.0–96.0
Wels	—	—	98.8–100	100	91.8–92.5	100	93.2–93.8	99.5–100	81.3–81.8	95.0–96.0

[a]Includes strains K11, K26, K58, and K64.
[b]Includes strains O20, O24, O52, and O64.
[c]Includes strains Ma32/94 (Genbank accession no. Z48235) and Ma370/94 (Z68191).
[d]Includes strains Tula/Moravia/5302Ma/94 (Z49915), Tula/Moravia/5294Ma/94 (Z48741), Tula/Moravia/5293Ma/94 (Z48574), Tula/Moravia/5286Ma/94 (Z48573), and Tula/Moravia/5302v/95 (Z69991).
[e]Includes strains Tula/175Ma/87 (Z30943), Tula/249Mr/87 (Z30944), Tula/76Ma/87 (Z30941), Tula/23Ma/87 (Z30945), and Tula/53Ma/87 (Z30942).

virus sequences, the Austrian TUL viruses all displayed a three nt deletion in the nucleocapsid gene that resulted in the loss of serine/alanine residue, which is found at position 252 of Russian TUL virus strains [Plyusnin et al., 1995; Sibold et al., 1995].

The three Austrian PUU virus sequences and eight TUL virus sequences were aligned with 38 hantavirus sequences retrieved from the GenBank sequence data base (Fig. 2). Unweighted maximum parsimony analysis of the data set was carried out by using PAUP. The large number of taxa necessitated use of a heuristic search, which yielded eight equally parsimonious trees. Each tree displayed a consistency index value of 0.34, indicating that there was considerable homoplasy (similarity in characters for reasons other than inheritance from a common ancestor; Hillis et al., 1996) in the data set. To reduce the contribution of homoplasy to the inferred phylogeny, characters were reweighted using the value of their rescaled consistency index. The heuristic search was repeated and yielded four equally parsimonious trees, a strict consensus of which is shown in Figure 2. Variation among the 4 trees was confined to terminal branches within the Russian PUU virus clade (K27, P360, cg1820) and within the clade containing TUL viruses from the Czech Republic (mor5302, mor94, mor93, mor5286, mor5302v). The PUU viruses occupy a highly supported (100% bootstrap support) clade with PUU90-13 at the basal position. The Austrian PUU viruses (Kl1, Kl6, P1) occupy a distinct, highly supported lineage within the PUU virus clade, but the phylogenetic relationship of this lineage to the Russian/Finnish/Swedish PUU lineage and PUU strain Berkel could not be fully resolved by this analysis because of weak bootstrap support (48%) for one of the intermediate branches within the PUU virus clade.

Within the well-supported TUL virus clade (Fig. 2), the TUL viruses from Austria, Slovakia, and the Czech Republic occupy a lineage that displays a sister relationship to a lineage containing TUL viruses from Russia. Within the Austrian/Slovakian/Czech lineage, the Austrian TUL sequences from Wels (O20, O24, O52, O64) occupy a distinct, well-supported basal sublineage. The Austrian TUL sequences from Korneuburg (K11, K26, K58, K64), on the other hand, exhibit well-supported (92%) monophyly with TUL viruses from Slovakia and the Czech Republic. There is weak bootstrap support (68%) for a sublineage containing the Korneuburg and Slovakian TUL sequences.

Because hantaviruses are notoriously difficult to isolate in tissue culture and often require a series of passages in laboratory-bred host rodents before they will grow in vitro [French et al., 1981; Elliott et al., 1994; Vapalahti et al., 1996], virus isolation was not attempted from any of the RT-PCR positive rodent tissues.

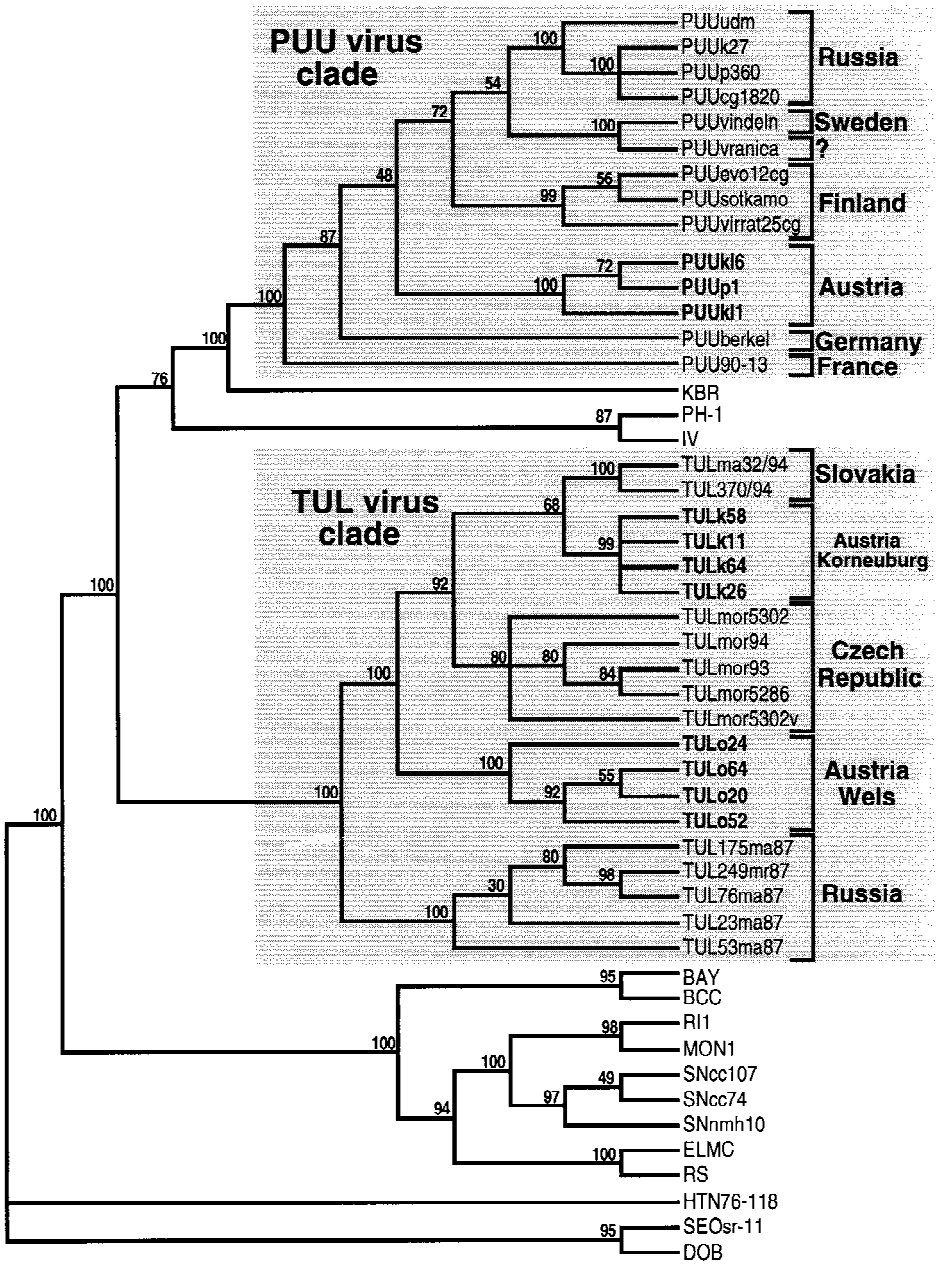

Fig. 2. The cladistic relationship of Austrian hantaviruses to other characterized hantaviruses. Small genomic RNA sequences of hantaviruses were retrieved from the GenBank sequences data base and aligned with the S RNA sequences of hantaviruses from Austrian rodents. The strain abbreviation, name, and GenBank accession number for each sequence are PUUudm (Puumala virus strain Udmurtia/894Cg/91, Z21497), PUUk27 (strain K27, L08804), PUUp360 (strain P360, L11347), PUUcg1820 (strain CG1820, M32750), PUUvindeln (strain Puu/Vindeln/L20Cg/83, Z48586), PUUvranica (strain Vranica, U14127), PUUevo12cg (strain Evo/12cg/93, Z30702), PUUsotkamo (strain Sotkamo, X61035), PUUvirrat25cg (strain PUUVirrat/25cg/95, Z69985), PUUberkel (strain Berkel, L36943), PUU90-13 (strain PUU90-13, U22423), KBR (Khabarovsk virus strain MF-43, U35255), PH (Prospect Hill virus, M34011 and X55128), IV (Isla Vista virus strain MC-SB-1, U31534), TULma32/94 (Tula virus strain Ma32/94, Z48235), TULma370/94 (strain Ma370/94, Z68191), TULmor5302 (strain Tula/Moravia/5302Ma/94, Z49915), TULmor5294 (strain Tula/Moravia/5294Ma/94, Z48741), TULmor5293 (strain Tula/Moravia/5293Ma/94, Z48574), TULmor5286 (strain Tula/Moravia/5286Ma/94, Z48573), TULmor5302v (strain Tula/Moravia/5302v/95, Z69991), TUL175ma87 (strain Tula/175Ma/87, Z30943), TUL249mr87 (strain Tula/249Mr/87, Z30944), TUL76ma87 (strain Tula/76Ma/87, Z30941), TUL23ma87 (strain Tula/23Ma/87, Z30945), TUL53ma87 (strain Tula/53Ma/87, Z30942), BAY (Bayou virus, L36929) BCC (Black CreekCanal virus, L39949), NY (New York virus strain Rl-1, U09488), MON (Monongahela virus strain Monongahela-1, U32591), SNcc107 (Sin Nombre virus strain Convict Creek 107, L33683), SNcc74 (strain Convict Creek 74, L33816), SNnmh10 (strain NM H10, L25784), ELMC (El Moro Canyon virus strain RM-97, U11427), RIOS (Rio Segundo virus strain RMx-Costa-1, U18100), HTN76-118 (Hantaan virus strain 76-118, M14626), SEOsr-11 (Seoul virus strain SR-11, M34881), and DOB (Dobrava/Belgrade virus strain 3970/87, L41916). Unweighted maximum parsimony analysis of the data set was carried out using PAUP, with characters optimized by accelerated transformation and gaps treated as missing data. The data set contained 612 characters, of which 385 were parsimony informative. A heuristic search was performed using stepwise addition, with random addition (10 replicates) and tree bisection-reconnection branch swapping. After the initial heuristic search, characters were reweighted using the maximum value of their rescaled consistency index and the heuristic search was repeated. The cladogram shown represents a strict consensus of 4 equally parsimonious trees. Bootstrap support for each internal node is displayed on the tree as a percentage of 1,000 heuristic search replicates. Hantavirus sequences obtained from Austrian rodents are indicated by boldface.

DISCUSSION

This report presents the first unequivocal evidence that two hantaviruses, PUU and TUL, are present in Austria. The PUU virus found in Austrian rodents from Carinthia apparently represents a new variety that is quite distinct from PUU viruses from other parts of Euorpe. The *Cl. glareolus* carrying PUU virus were trapped in the region of Carinthia (northern Lavanttal Valley), where most of the Austrian NE cases have been reported [Aberle et al., 1996]. Unfortunately, clinical specimens from Austrian NE cases were not available for this study. Typing of PUU viruses from clinical cases of NE will be necessary to determine if this new PUU genotype is actually responsible for disease in Austria.

The presence of TUL virus in Austrian *M. arvalis* is not too surprising, given the detection of TUL virus in rodents from the Czech Republic and Slovakia [Plyusnin et al., 1995; Sibold et al., 1995]. What is somewhat unexpected is the presence of two genetically distinct variants of TUL virus in Austrian rodents. It appears that the genetic distances between the TUL virus variants from these three countries correlates with the geographic distance between the regions where the infected rodents were captured (Fig. 1). The TUL virus sequences from Korneuburg and Malacky, Slovakia are the least divergent (2% to 3% nt divergence) and these two sites are the closest together (approximately 65 km). Korneuburg is approximately 100 km southwest of the region where *M. arvalis* yielding the Czech TUL virus sequences were trapped [Plyusnin et al., 1995], and these sequences differ by 3% to 4%. The sites in Slovakia and the Czech Republic are about 80 km apart, and there is approximately 4% divergence in the nucleocapsid gene sequences of strains from these areas [Plyusnin et al., 1995]. Wels is approximately 170 km west of Korneuburg; rodents trapped in this area harbor TUL virus strains that differ by 6%–8% at the nucleotide level from strains from Korneuburg, Slovakia, and the Czech Republic. In all the central European TUL virus sequences analyzed in this study, the observed substitutions were almost exclusively synonymous. Previously, distinct geographic clustering of TUL virus strains has been demonstrated for viruses from distantly geographic areas (i.e., Czech Republic and Russia; Plyusnin et al., 1995). The data presented in this study suggest that a gradient of sequence divergence can be seen in TUL virus strains when *M. arvalis* populations are sampled over smaller geographic areas.

In this study, antibody that reacts with PUU virus antigen by ELISA was found in several *A. flavicollis*, *A. sylvaticus*, and *Mus musculus* specimens. In addition, hantavirus antigen was questionably detected in the lung tissues of a single seropositive *A. sylvaticus*. Hantavirus genomes were not detected by RT-PCR in a small number of *Apodemus* spp. and *Mus musculus* that were examined. DOB virus has been isolated from *A. flavicollis* captured in neighboring Slovenia, and hantavirus antibody or antigen has been detected in Slovenian *A. flavicollis* and *A. sylvaticus* [Avsic-Zupanc et al., 1992]. A DOB or HTN-like virus could be present in Austrian *Apodemus* spp. that might escape detection by our assays since they were optimized for PUU virus (ELISA, antigen detection) or PUU, TUL, and Prospect Hill viruses (RT-PCR, nested PCR). The possible identity of the hantavirus infecting *Mus musculus* is unpredictable. Two hantavirus isolates, Leakey and POZ-M1, have been reported from *Mus musculus* [Baek et al., 1988; Diglisic et al., 1994]. Puthavantha and co-workers [1992,1993] later showed that one stock of Leakey virus was a mixture of a Seoul virus and laboratory strain of PUU virus originating from Finland (strain Yanagahara). Xiao and co-workers [Xiao et al., 1994] found that another Leakey virus stock was a PUU virus that was genetically identical to the Swedish PUU strain Hällnäs-B1, and determined that POZ-M1 was a PUU virus that was almost identical to Hällnäs-B1. Thus it appears that the Leakey and POZ-M1 isolates represent laboratory cross-contaminants rather than true isolates. None of the seropositive *M. musculus* tested in this study contained PUU virus antigen. The antibody detected in *M. musculus* could indicate the presence of a distinct hantavirus in *Mus* but could also be the result of immunizing infection of hantaviruses from sympatric rodent species. Whether or not *M. musculus* harbors its own hantavirus remains to be determined.

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